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Fungal control of early-stage bacterial community development in decomposing wood

Sarah R. Christofides*, Jennifer Hiscox, Melanie Savoury, Lynne Boddy, Andrew J. Weightman

Cardiff School of Biosciences, Cardiff University, CF10 3AX, Wales, UK

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ABSTRACT

The earliest stages of bacterial colonisation of wood have received little attention, particularly with respect to how the colonisation process may be affected by the presence of wood-decay fungi. This study used 16s rRNA gene sequencing to examine the bacterial community in wood that had been incubated in the field for 14 or 84 d, either in wood uncolonised by fungi or pre-colonised by *Vuilleminia comedens, Trametes versicolor* or *Hypholoma fasciculare*. All three fungal species significantly delayed bacterial colonisation of the wood. *V. comedens* and *H. fasciculare* also reduced bacterial OTU richness and altered bacterial community composition, increasing the relative abundance of *Burkholderiales* and reducing the proportion of *Enterobacteriaceae* and *Bacteroidetes*. Wood that had not been pre-colonised showed seasonal differences between autumn and spring: bacterial richness increased between 14 d and 84 d in the spring, but not in the autumn. Community composition at 84 d in spring was also different to the other time points, with reduced dominance of *Gamma-proteobacteria. Archaea* were also detected in nearly a third of samples, but with no apparent pattern, and always at low abundances.

1. Introduction

Decomposing wood is colonised by a mosaic of interacting fungal species that overall form a dynamic successional series, from the R (ruderal) and S (stress-tolerant) species present during early decay, through a series of increasingly competitive decomposer species (Boddy, 2001). At late stages of decay, these highly competitive fungi are in turn often replaced by stress-tolerant species. Bacterial succession in wood is much less well understood, although there have been a number of correlative studies determining the bacterial community in wood of different decay classes (Hoppe et al. 2015; Rinta-Kanto et al. 2016; Kielak et al. 2016). The very earliest stages of wood colonisation by bacteria have rarely been examined (Sun et al. 2014).

Although bacteria are often said to be the earliest colonisers of wood, influencing which fungi can subsequently establish (Greaves, 1971; de Boer and van der Wal, 2008; Sun et al. 2014), it is likely that bacteria colonising wood almost always encounter a fungal community that has already developed. Wood-decay fungi are latently present in functional sapwood, and rapidly colonise wood once the water content drops to a favourable level (Boddy, 2001). Consequently, fungal decay begins in the canopy, and wood is usually well colonised before it falls to the forest floor. Whilst bacterial saprotrophs may likewise live endophytically in wood, a large component of the bacterial community in dead wood probably originates from soil (Johnston et al. 2016), and thus the bacteria are secondary colonisers. There is evidence that wood-decay fungi exert active selection over bacteria, raising the possibility that fungi act as 'gatekeepers' determining which bacteria may enter a resource (Johnston et al. 2018). The time-scales over which this operates are unknown (Johnston et al. 2016).

In a study in which wood pre-colonised by specific fungi decayed on the forest floor (Johnston et al. 2018), distinct bacterial communities were associated with cord-forming basidiomycete fungi, which are highly competitive late-secondary colonisers. However, of the original fungal pre-colonisers, only one retained its territory over the 1-y duration of the experiment; the rest were replaced by other fungi through natural succession. This meant that, although bacteria were often correlated with particular fungi, whether the relationship was causative largely could not be

* Corresponding author.
E-mail address: ChristofidesS@cardiff.ac.uk (S.R. Christofides).

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determined. It is an open question as to whether non-cord-forming, less competitive fungi are also able to manipulate bacterial communities. These less competitive, earlier-stage colonisers are typical of the fungal community that bacteria would encounter in newly-fallen wood.

In addition to biotic determinants, microbial communities are highly influenced by seasonal variation and accordingly show distinct patterns of community composition and activity (López-Mondéjar et al. 2015; Žifčáková et al. 2016). Seasonality is particularly pronounced in temperate climates, where water availability, temperature and daylight are highly influenced by the time of year. Little is known of seasonal effects on bacteria in wood, but there are indications that season may be important: a model of nitrogen fixation in wood predicted considerable seasonal changes, with different abiotic factors becoming limiting at different times of year (Hicks et al. 2003). Forest litter and soil also show seasonal differences in bacteria:fungus ratios, functional activity and horizon-specific bacterial community composition (López-Mondéjar et al. 2015; Žifčáková et al. 2016).

The present study investigates the influence of fungal pre-colonisation and season on bacterial community composition in decomposing wood, during the earliest stages of colonisation. A manipulative field experiment was designed to examine bacterial colonisation of wood pre-colonised with fungi, in both spring and autumn. Four predictions were tested: (1) the fungal species within the wood would alter both the diversity and composition of the bacterial community, with competitive fungi hosting a less diverse bacterial community; (2) this effect would become increasingly apparent over time; (3) the fungal influence would be most prominent in the autumn, when many fungi are active; and (4) within-season variability would be evident within the bacterial community, but that this stochastic variation would be secondary to the fungal and seasonal effects above.

2. Materials and methods

2.1. Overview

Wood blocks were colonised with wood-decay fungi in the laboratory and then placed on the forest floor for 14–84 d to allow bacterial colonisation. The fungi were specifically chosen to encompass a range of successional stages and competitive abilities (Table 1). The experiment was repeated at three starting dates in both spring and autumn, *i.e.* 6 times. Bacterial communities were then characterised by amplicon sequencing. The entire experiment used 192 samples (4 wood treatments (uncolonised + 3 fungal colonisers) x 4 replicates x 2 seasons x 3 starting points x 2 time periods).

2.2. Field experiment

Blocks of kiln-dried beech wood (*Fagus sylvatica*; 3 × 3 × 3 cm) were sterilised by triple autoclaving, leaving at least 24 h between runs. The sterilised blocks were sealed in 1000 ml plastic tubs (Cater4you, UK) containing a base covering of malt agar colonised by either *Hypholoma fasciculare* (HfGTWV2), *Trametes versicolor* (TvAW-HxFP) or *Vuilleminia comedens* (VcWVJH1) (Table 1). Blocks were left to colonise for a minimum of 3 months in the dark at 20 °C. 25% of blocks were kept sterile as controls, and stored at −20 °C until placed in the field.

Before being placed in the field, each block was scraped free of surface mycelium, and paired with another colonised by the same fungus with vessel ends touching. Control (uncolonised) blocks were likewise paired with each other. Pairs were held together with plastic-coated wire (Fig. S1). This double-block set-up was necessary as the samples were also being used in a second experiment involving interspecific pairings. Blocks were placed in a random-position grid in mixed woodland, with *F. sylvatica* the dominant species (Whitestone Woods, Tintern, lat. 51.72 long. −2.69). Blocks were set out in 2015, at 2-week intervals on three dates in spring (4th May, 18th May and 1st June) and three dates in autumn (15th Oct, 29th Oct and 12th Nov). Set-outs were staggered within seasons to capture stochastic variability in time, creating three 'sets' per season. Each 2 × 2 m grid square contained two block pairings of each treatment, secured with a tent peg through a loop in the wire. Half the samples from each square were collected after 2 weeks, and the remainder after 12 weeks (4 replicates per treatment per time point). Blocks were returned to the lab and processed within a few hours of collection. Each block was split into quarters with a sterile chisel, wrapped in sterile foil and flash-frozen in liquid N_2 for storage at −80 °C. One quarter of each block was left unfrozen for fungal re-isolation to determine pre-coloniser persistence: four small chips were removed from the interior of each block (*i.e.* 8 per 2-block sample) under aseptic conditions and isolated onto 2% malt agar. Outgrowing mycelium was identified as pre-coloniser or non-pre-coloniser by morphology.

2.3. Molecular analysis

Frozen blocks were drilled with a 4 mm bit under aseptic conditions to create swarf (wood dust) for DNA extraction. 0.3–0.5 g swarf from each block was immediately added to a PowerSoil® bead tube and re-frozen at −20 °C. Extraction negative controls were performed by running the drill over an open tube. DNA was extracted following the MoBio PowerSoil® kit protocol (Carlsbad, CA USA), with the vortex step replaced by 3 × 20 s in a MP FastPrep®-24 bead beater at 4 m s^{-1}.

The presence of bacterial DNA was verified by PCR with primers 27F (AGAGTTTGATCMTGGCTCAG) (Weisburg et al. 1991) and 907R (CCGTCAATTCCTTTGAGTTT) (Lane et al. 1985). Each 50 μl reaction contained 1 μl DNA extraction, 200 nM each primer (MWG Eurofins, Ebersberg, Germany), 5 μg BSA (Promega, WI, USA) and 0.025 U μl^{-1} Taq polymerase (PCR Biosystems, London, UK) in 10 μl supplied buffer. Amplification was performed in a Dyad DNA Engine Peltier thermal cycler (Bio Rad, Herts, UK) (95 °C for 2 min, 35 x [94 °C for 30 s, 52 °C for 30 s, 72 °C for 1.5 min] increasing by 1 s $cycle^{-1}$, 72 °C for 5 min). Samples that failed to amplify were retested at 1:10 and 1:100 dilutions to ascertain that the reaction was not affected by inhibitors. A subset of failed samples was tested using 5 μl DNA template, to no avail, and also screened with 515F-806R primers (see below) to check that the failure was not due to fragmented DNA.

For samples that successfully amplified, next-generation

Table 1
Fungal species used in the experiment. All fungi are white-rot wood-decay basidiomycetes from the Cardiff Culture Collection.

Name	Strain	Family	Ecological strategy	Acronym
Hypholoma fasciculare	GTWV2	Strophariaceae	Late stage secondary/tertiary colonist; cord former	Hf
Trametes versicolor	AW-HxFP	Polyporaceae	Early-mid stage secondary colonist	Tv
Vuilleminia comedens	WVJH1	Corticiaceae	Primary colonist	Vc

sequencing and library construction was delivered via the BBSRC National Capability in Genomics and Single Cell (BB/CCG1720/1) at Earlham Institute by members of the Genomics Pipelines Group. Each sample was amplified in duplicate with primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al. 2011; Kozich et al. 2013) and the products pooled. Each 25 μl reaction contained 2 μl each of the forward and reverse primers (Kozich et al. 2013) at 2.5 μM, 0.1 μl Kapa 2G Robust polymerase (Kapa Biosystems KK5005) and 0.5 μl 10 mM dNTPs; the PCR program was as follows: 94 °C for 3 min, 25 x [94 °C for 45 s, 55 °C for 15 s, 72 °C for 30 s], 72 °C for 3 min. This primer pair targets the V4 region of the 16S rRNA gene and is widely used for environmental community characterisation, notably in the Earth Microbiome Project (Caporaso et al. 2011; Walters et al. 2016). Amplicons were sequenced on an Illumina MiSeq (v2, 2 × 250 bp) with Nextera XT assay chemistry.

2.4. Sequence analysis

Paired-end reads were merged using QIIME 1.9.1 (Caporaso et al. 2010). Low-quality sequences were removed with USEARCH v9.0.2132 (1 max expected error, minimum length 250 bases) (Edgar, 2010). Chimeras were identified and removed using USEARCH 61 implemented in QIIME. A number of over-length sequences were still present after chimera removal, so any sequences longer than 257 bases were removed prior to open reference OTU picking at 97% similarity against the Greengenes 16S rRNA gene database (DeSantis et al. 2006). Singleton OTUs (*i.e.* those containing only one sequence) were excluded. To check for fungal amplification, OTU picking was repeated again the SILVA_119 16S/18S rRNA gene database (Pruesse et al. 2007; Quast et al. 2013). No fungal sequences were detected, so the Greengenes OTUs were used for subsequent analysis. Sequences are archived at the European Nucleotide Archive (ENA) under accession number PRJEB87091. All other data associated with the analysis is available on ResearchGate under DOIs 10.13140/RG.2.2.22140.18567, 10.13140/RG.2.2.24656.76802, 0.13140/RG.2.2.14590.43848 and 10.13140/RG.2.2.19623.60324.

2.5. Statistical analysis

All analysis was performed in R (R Development Core Team, 2011) using RStudio (RStudio Team, 2016) and packages *dplyr* (Wickham and Francois, 2016), *lsmeans* (Lenth, 2016), *MASS* (Venables and Ripley, 2002), *metacoder* (Foster, 2016) and *vegan* (Oksanen et al. 2016). All R code to reproduce the analyses is available as an R markdown file at github.com/ecologysarah/time-series (Allaire et al. 2016).

A Bernoulli GLM (conditional log-log link) was used to model the presence/absence of bacteria in the samples versus season, pre-coloniser and time in the field. Bacteria were counted as present if the original DNA extraction produced a visible amplification product after 35 cycles.

Although the experimental design was fully crossed, the variable success in bacterial amplification meant that many treatments were absent from the sequencing dataset or had <3 replicates. Therefore, it was decided to break the data into subsets which were analysed separately to test particular hypotheses. Although this approach is not ideal, as it increases the number of models run, it was considered preferable to attempting a single, highly imbalanced and nested model. Only the control samples contained sufficient replicates to test the effects of season and length of time in the field; therefore, the 'time' subset consisted of all control samples, in both spring and autumn, at 14 and 84 d. Each of the pre-coloniser treatments could only be analysed at 84 d and in one season: for *T. versicolor* and *H. fasciculare* that season was spring, whilst for *V comedens* it was autumn. Given that the time dataset showed significant differences between seasons (see results), pre-colonised samples from different seasons were not combined. *T. versicolor* and *H. fasciculare* were compared to 84-d control samples from the spring ('spring' subset), and *V comedens* to 84-d control samples from the autumn ('autumn' subset). The autumn subset was the only dataset where comparison could be made between three different set-outs. A summary of which samples were used for which analyses is given in Table S1. Sequencing data were not rarefied prior to analysis for the reasons discussed in Johnston et al. (2018). In the current dataset, depth was not homoscedastic among predictors, but it was nonetheless deemed better to take this into account explicitly during the analysis rather than implicitly by rarefying. To check for robustness of the results, the data were rarefied to 3708 sequences per sample (the depth of the smallest sample passing QC) and the analysis was re-run. The results were identical aside from minor discrepancies, and the full rarefied results are given in Supplementary Material 1.

Hypotheses pertaining to bacterial community composition were tested by permutation ANOVA (PERMANOVA; 999 permutations) on a Bray-Curtis distance matrix (Anderson, 2001). *Post hoc* tests were approximated by subsetting the dataset into pairwise combinations and running PERMANOVA on each (Hiscox et al. 2016). In this instance, no *P*-adjustment was performed due to the limited number of comparisons involved. The predictor variables for the time dataset were sequencing depth (continuous), season (categorical), day (categorical) and the season-day interaction. Pairwise comparisons were tested for a difference between seasons at 14 d and at 84 d, as dispersions were equal for these comparisons but not *vice versa*. The spring and autumn dataset analyses contained depth and pre-coloniser as predictors; the autumn dataset also included set and the pre-coloniser-set interaction.

To visualise community differences, the data were ordinated using 4 axes of non-metric dimensional scaling (NMDS) on a Bray-Curtis distance matrix. Sequencing depth could be isolated on axis 2 (Fig. S2a). To check for artefacts introduced by overdispersion (Warton et al. 2012), qualitative comparison was made with principal components analysis using a Hellinger transformation (Fig. S3). The taxonomic composition of each treatment was also visualised down to family level using heat trees (Foster et al. 2017).

Differences in OTU richness were modelled using negative binomial generalised linear models, to account for overdispersion in the data. A square root link was added for the time model to improve parametric assumptions. The model for each subset contained the same predictors as the PERMANOVA. Where interactions were included, likelihood ratio tests were used to assess their overall significance. To plot richness whilst controlling for the influence of sequencing depth, the square-root transformed richness values were regressed against sequencing depth. The residuals were back-transformed and plotted in the place of raw richness values.

3. Results

3.1. Patterns in bacterial presence/absence

Bacteria were amplified from 75 out of 188 original DNA extractions. Detectable bacterial presence was significantly less common in pre-colonised wood samples than in controls, and in 14-d exposures compared to 84 d (Fig. 1; Table 2; Table S2). Season had no effect on the probability of bacterial detection. All the fungal pre-colonisers decreased the likelihood of bacterial presence, but *T. versicolor* had the largest negative effect, followed by *H. fasciculare* and then *V. comedens*. All fungal pre-colonisers

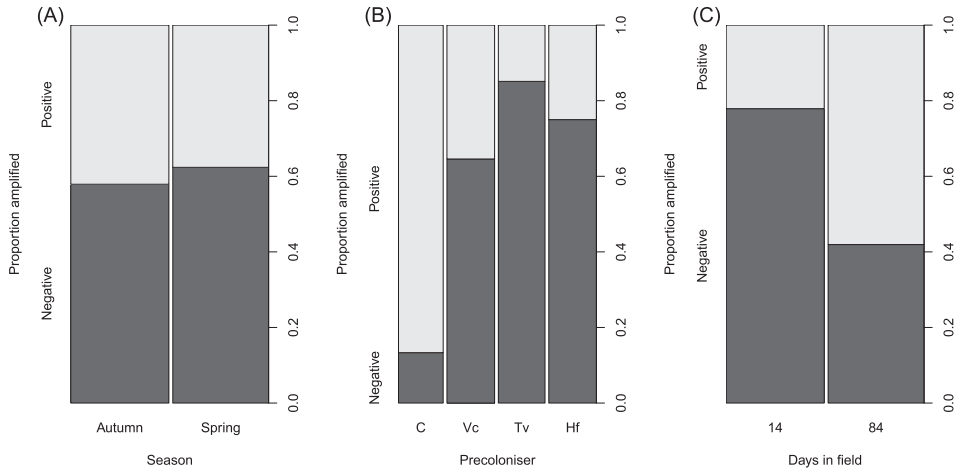

Fig. 1. Probability of detection of bacterial presence (measured by PCR amplification) in fungus-colonised wood blocks incubated in the field, separated by: (A) season of incubation; (B) pre-coloniser identity; or (C) length of time in field. Pre-colonisers were *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf) or uncolonised controls (C).

Table 2
Results of Bernoulli GLM on the presence of detectable bacteria in fungus-colonised wood.

	Coefficient	Std. error	*z*	Pr(>|*z*|)
Intercept	0.539	0.293	1.84	0.066
Season: spring	−0.398	0.286	−1.39	0.164
Species: Hf	**−3.59**	**0.682**	**−5.26**	**<0.001**
Species: Tv	**−4.38**	**0.738**	**−5.94**	**<0.001**
Species: Vc	**−3.03**	**0.655**	**−4.62**	**<0.001**
Day: 84	**2.81**	**0.609**	**4.61**	**<0.001**

Null deviance: 252.89 on 187 degrees of freedom; residual deviance: 140.48 on 182 degrees of freedom; $R^2 = 0.555$. Reference levels are autumn for season; control for species; and 14 d for day. Pre-colonisers are *V. comedens* (Vc), *T. versicolor* (Tv) and *H. fasciculare* (Hf). All numbers are given to three significant figures. Significant differences are shown in bold.

retained a strong presence in the wood blocks as determined by re-isolation (Fig. S4), although *V. comedens* was starting to lose territory by 84 d (Fig. S4B). Isolations from the control blocks displayed a mixture of fungi and bacteria in all samples. Morphological identification indicated that these fungi were largely fast-growing, R-selected, non-lignocellulolytic fungi with few occurrences of probable wood decayers.

3.2. Preliminary analysis of sequence data

7 561 110 reads passed quality filtering, and were grouped into 15 830 OTUs. The three extraction kit negative controls retained 3, 5 and 6 reads, respectively, so were excluded from further analysis. One 84-d *T. versicolor* pre-colonised sample from the spring yielded only 7 reads, and one 84-d *V. comedens* pre-colonised sample from the spring yielded only 507 reads; both were also excluded. All remaining samples contained more than 3 700 reads. There was no clustering by extraction kit lot number (Fig. S2b). Rarefaction curves showed that OTU diversity almost saturated for many of the samples, particularly those pre-colonised with *H. fasciculare* or *V. comedens* (Fig. S5). 15 251 OTUs were assigned to *Bacteria*, 19 to *Archaea* and 560 were unassigned at the Domain/Kingdom level. Although subsequent analysis refers to 'bacteria' for simplicity, all OTUs were included.

3.3. Presence of Archaea in wood

Archaea were detected in 20 wood block samples (30%), apparently distributed randomly across treatments. Archaeal reads never accounted for more than 0.8% of the read counts for any given sample. 13 of the 19 archaeal OTUs were *Euryarchaeota*, and of these 6 were *Methanomicrobia* and a further 6 were *Methanobacteria*. However, the most abundant archaeal OTU, both in read counts and number of observations, was assigned to the *Parvarchaeota*.

3.4. Temporal effects on the bacterial community development in wood

Comparisons between time points could only be made among the control samples. PERMANOVA showed a significant interaction between season and length of time in the field. Pairwise comparisons revealed that the seasons were significantly different at 84 d ($F = 18.3$, term $R^2 = 0.515$, $P = 0.001$), but not at 14 d ($F = 1.25$, term $R^2 = 0.051$, $P = 0.202$) (Fig. 2B). OTU richness likewise had a significant interaction between season and field duration ($LR = 7.14$, $Pr = 0.008$); richness was higher at 84 d than at 14 d in the spring, but not in the autumn (Fig. 3B–D).

Proteobacteria dominated at all time points. *Pseudomonadaceae* were dominant in the 14-d samples, along with *Burkholderiales* (particularly *Oxalobacteraceae*) (Fig. 4). The 84-d samples were enriched in *Bacteroidetes*, particularly in the spring, whilst the autumn samples were heavily dominated by *Enterobacteriaceae*. The spring 84-d samples showed increased abundance of *Alphaproteobacteria*.

3.5. Fungal effects on the bacterial community

Comparisons between the bacterial communities in the control wood blocks and those pre-colonised by *T. versicolor* or *H. fasciculare* could only be made among the 84-d spring samples, while comparison between *V. comedens* pre-colonised wood and the controls could only be made among the 84-d autumn samples. PERMANOVA showed a significant difference between wood with different pre-coloniser treatments in both subsets ($F = 2.73$, term $R^2 = 0.199$, $P = 0.003$ for the spring data, $F = 18.5$, term $R^2 = 0.401$, $P = 0.001$ for the autumn data) (Fig. 2A). Pairwise comparisons among the spring dataset revealed a significant difference between the bacterial community in *T. versicolor* and *H. fasciculare* pre-colonised wood ($F = 3.18$, term $R^2 = 0.251$, $P = 0.001$), but neither the bacterial community associated with *T. versicolor* ($F = 0.815$, term $R^2 = 0.05$, $P = 0.507$) nor the bacterial community associated with *H. fasciculare* ($F = 1.426$, term $R^2 = 0.067$, $P = 0.200$) was

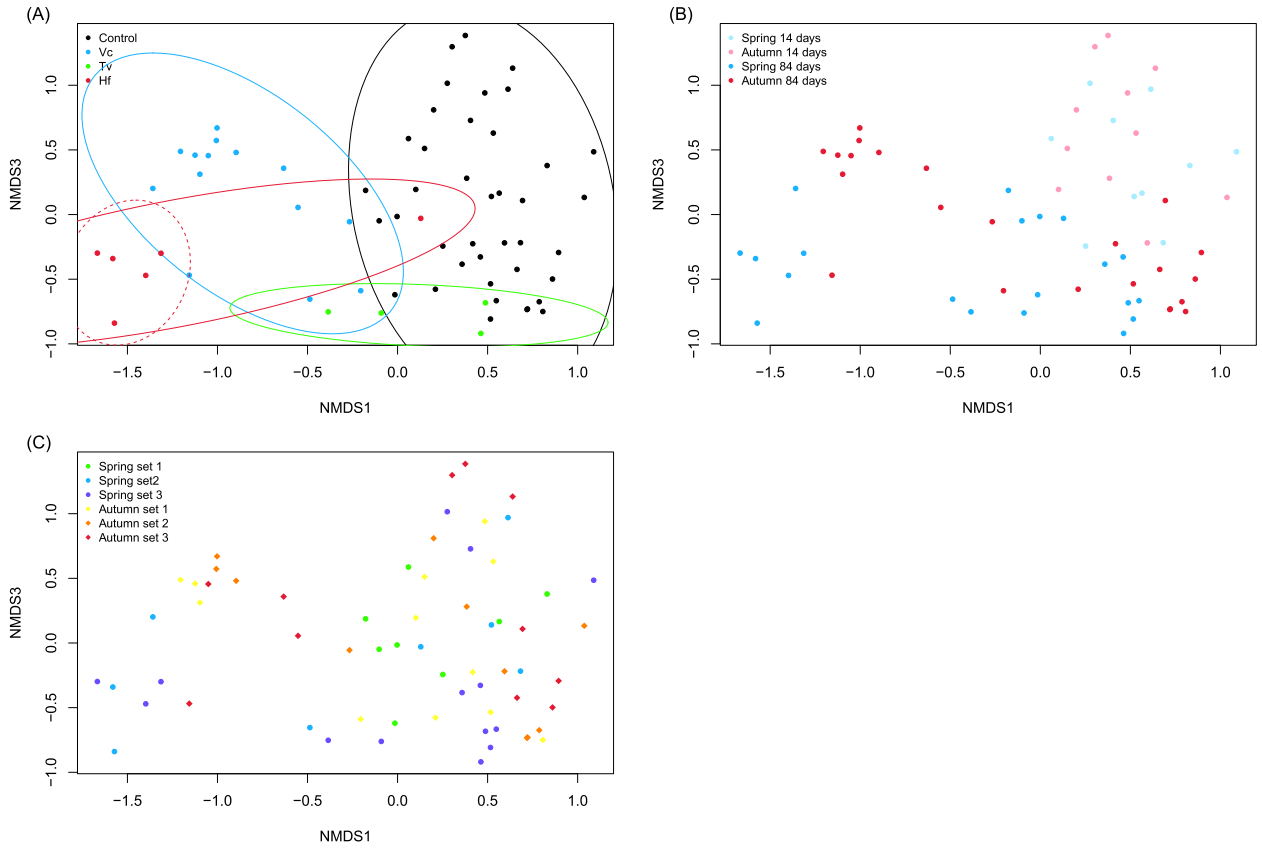

Fig. 2. NMDS ordination of the bacterial community in fungus-colonised wood blocks. Points are coloured by: (A) fungal pre-coloniser *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf) or uncolonised controls (C); (B) season of field incubation and length of time in field; or (C) set-out date. In (A), 95% confidence ellipses are shown for each pre-coloniser (solid lines), and for *H. fasciculare* excluding one outlying point (dashed line).

significantly different from that in the controls. The autumn dataset revealed no significant influence of set-out date (set), nor any interaction between wood block set-out date and pre-coloniser (Fig. 2C).

Bacterial OTU richness in the spring samples was significantly lower in *H. fasciculare* pre-colonised blocks than in the controls ($z_{14,17} = -2.46$, $Pr(>|z|) = 0.014$), but there was no difference in bacterial community richness between *T. versicolor* pre-colonised samples and the controls ($z_{14,17} = 1.26$, $Pr(>|z|) = 0.207$) (Fig. 3A). In the autumn samples, there was a significant interaction between pre-coloniser species and set-out date ($LR = 8.94$, $Pr = 0.011$) (Fig. 3E). OTU richness in *V. comedens* pre-colonised wood was significantly lower than the controls in sets 1 and 3, but not in set 2; the set 2 controls were also significantly different from the controls in sets 1 and 3 (Table 3).

H. fasciculare and *V. comedens* pre-colonised wood blocks were both enriched in *Burkholderiales*, particularly *Burkholderiaceae*, compared with the controls (Fig. 5). *T. versicolor* pre-colonised wood showed little difference in bacterial community composition to the controls, apart from a slight reduction in *Beta-proteobacteria* and *Bacteroidetes*. The relative abundance of *Acidobacteria* showed little variation across any of the treatments.

4. Discussion

This study demonstrates, for the first time, that wood-decay fungi not only alter bacterial communities but also significantly delay their establishment within wood. When bacteria do colonise the wood, some fungi also affect community composition in a species-specific manner. This study also provides the first indications of the timescale over which the earliest stages of bacterial colonisation operate in wood arriving on the forest floor.

4.1. Wood-decay fungi can exclude bacteria from their resource

Detectable bacteria were strikingly higher in the control samples than pre-colonised samples. In most of the pre-colonised wood samples, bacteria had not colonised to a detectable extent after 14 d, and detection remained patchy even at 84 d. By contrast, bacteria were consistently present in the controls, albeit at low levels. This indicates that wood-decay fungi can delay bacterial colonisation, probably due to the selection pressure that they exert over the bacterial community (Folman et al. 2008; Hervé et al. 2014; Johnston et al. 2018). Negative PCR results do not necessarily indicate that bacteria were truly absent but rather that they were too scarce to detect, as bacteria were obtained by isolation from samples that were negative by PCR. The control samples were not fungus-free, but the fungal colonisers were poorly established, with high representation of fast-growing, R-selected, non-lignocellulytic fungi rather than wood decayers. Folman et al. (2008) also reported difficulty in obtaining 16S rRNA gene PCR products from fungus-colonised wood in microcosms, although with no effect of increasing time. This was corroborated by microscopy and plate counts, which demonstrated that the fungi reduced both the number of bacterial cells present in the wood and the proportion which could be obtained by cultivation (Folman et al. 2008).

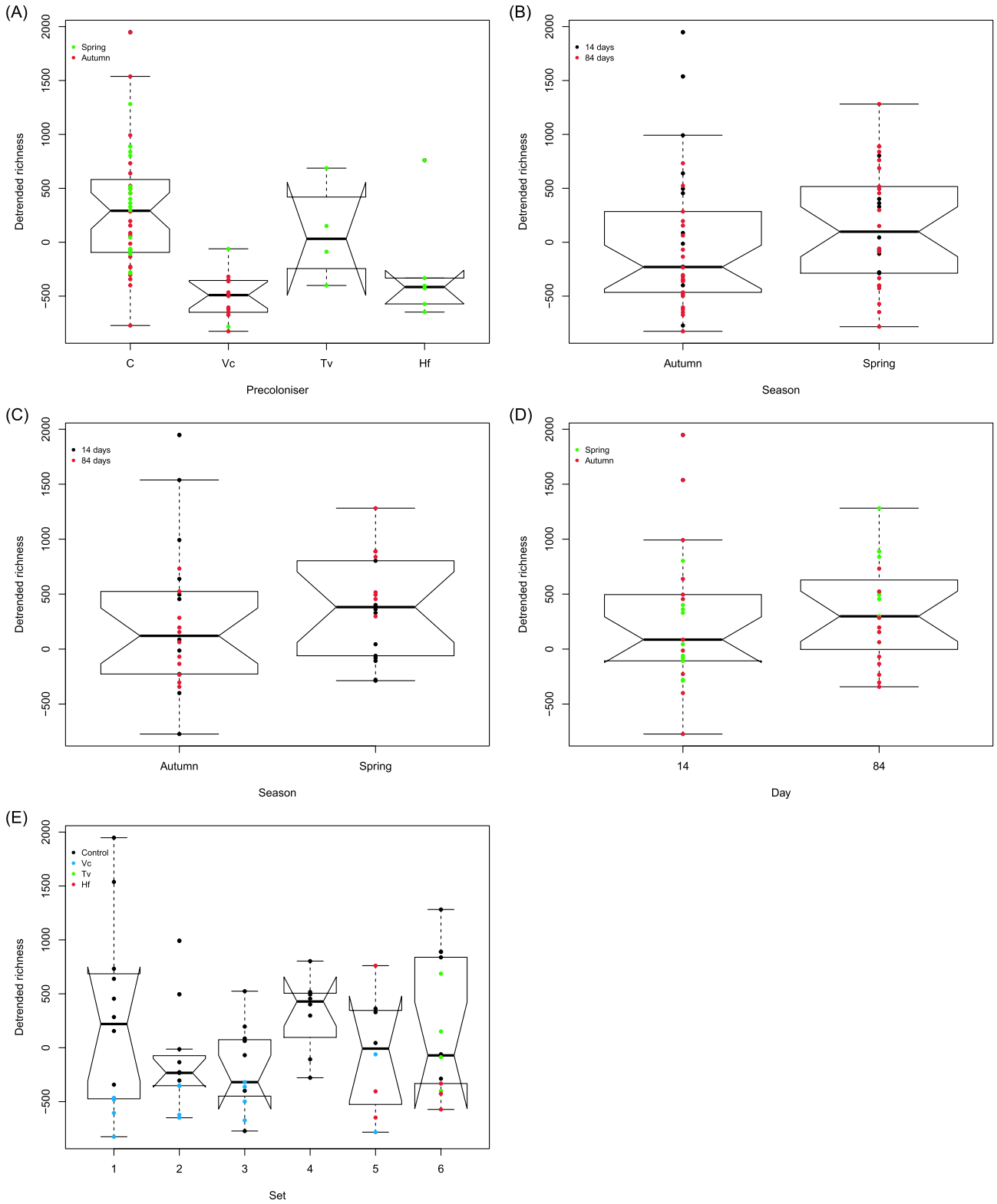

Fig. 3. Overall bacterial OTU richness in fungus-colonised wood blocks. Residuals from a model to correct for sequencing depth (the *y*-scale is therefore arbitrary), broken down by: (A) pre-coloniser identity (*V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf) or uncolonised controls (C)), coloured by season; (B) all samples split by season of field incubation, coloured by length of time in the field; (C) control samples only, split by season of field incubation, coloured by length of time in the field; (D) control samples only, split by length of time in the field, coloured by season; (E) set-out date, coloured by pre-coloniser (1–3 represent autumn set-outs, 4–6 are spring). Individual data points are overlaid on boxplots. Notches on boxplots represent 95% confidence intervals; where these extend beyond the quartiles, 'hinges' appear on the plot.

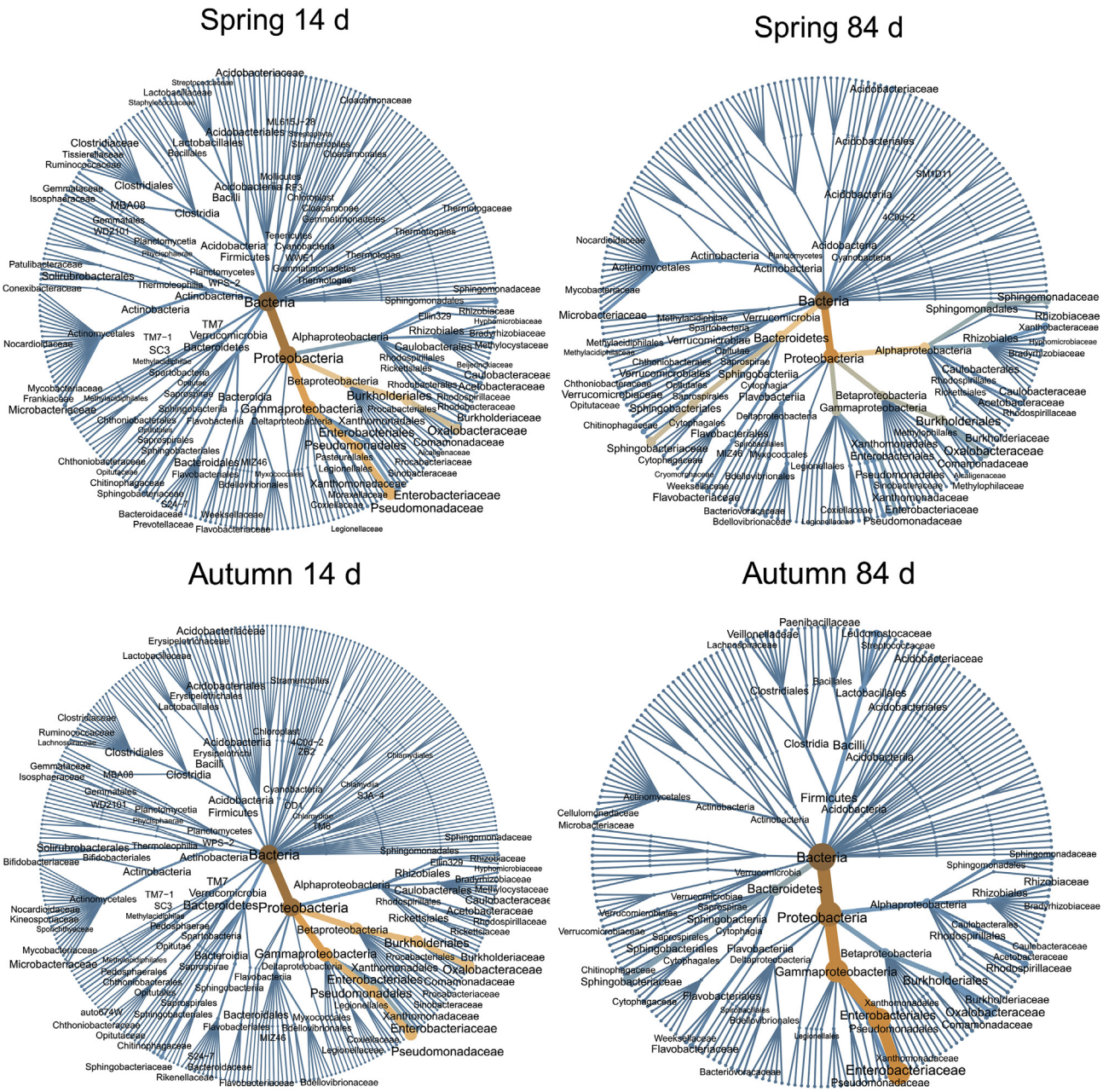

Fig. 4. Bacterial community composition in control wood blocks (without fungal pre-colonisation), broken down by season and length of time in the field. Node colour and size on the heat trees represent relative abundance for that taxon; relative read abundance is on an arbitrary scale where each sample sums to 100. OTUs assigned to *Archaea* or unassigned at the Domain/Kingdom level are excluded for ease of visualisation.

4.2. *Primary colonising fungi manipulate bacterial communities*

The distinct bacterial community in *V. comedens* pre-colonised wood blocks showed that primary colonising, early-decay fungi can nonetheless influence which bacteria can establish. This was not consistent with the suggestion in Johnston et al. (2018) that only highly competitive cord-forming fungi select bacteria, and raises the question why distinct bacterial communities were not apparently associated with the less competitive fungi in the results reported in Johnston et al. (2018). One possibility is that cord-forming fungi all select for similar bacteria, whilst less competitive fungi have more varied bacterial communities. A more likely explanation is that fungal influence on bacterial community development is linked to the length of time that the fungus occupies the resource. Highly competitive fungi can hold a resource for a long time and clearly establish their influence over it, whereas a resource that frequently changes fungal coloniser is likely to exhibit a patchwork of legacy effects (Hiscox et al. 2015; Leopold et al. 2017).

In the present study, the effects of pre-colonisation by the highly competitive cord-former *H. fasciculare* did not significantly alter bacterial communities compared to the control, but this was probably due to the influence of one outlier, as all the other *H. fasciculare* samples formed a tight cluster (Fig. 2A). Despite differences in their competitive ability, pre-colonisation by *V. comedens* and *H. fasciculare* reduced the observed bacterial

Table 3
Post-hoc comparisons of bacterial OTU richness between wood blocks colonised with *V. comedens* (Vc) and control blocks, at 84 d across three set-outs staggered 2 weeks apart.

Contrast		Coefficient	Std. error	*z*	*P*
Set 1 control	**Set 2 control**	**0.348**	**0.106**	**3.28**	**0.013**
	Set 3 control	0.043	0.097	0.439	0.998
	Set 1 Vc	**0.706**	**0.098**	**7.24**	**<0.001**
	Set 2 Vc	**0.606**	**0.099**	**6.11**	**<0.001**
	Set 3 Vc	**0.645**	**0.108**	**5.99**	**<0.001**
Set 2 control	**Set 3 control**	**−0.305**	**0.100**	**−3.06**	**0.027**
	Set 1 Vc	**0.359**	**0.100**	**3.57**	**0.005**
	Set 2 Vc	0.258	0.100	2.57	0.104
	Set 3 Vc	**0.297**	**0.102**	**2.92**	**0.042**
Set 3 control	**Set 1 Vc**	**0.664**	**0.093**	**7.17**	**<0.001**
	Set 2 Vc	**0.563**	**0.093**	**6.08**	**<0.001**
	Set 3 Vc	**0.602**	**0.096**	**6.30**	**<0.001**
Set 1 Vc	Set 2 Vc	−0.101	0.093	−1.08	0.890
	Set 3 Vc	−0.062	0.096	−0.639	0.988
Set 2 Vc	Set 3 Vc	0.039	0.095	0.412	0.999

Comparisons are derived from a negative binomial general linear model. Results are given on the log (not the response) scale. *P* value adjustment: Tukey method for comparing a family of 6 estimates. All numbers are given to three significant figures. Significant differences are shown in bold.

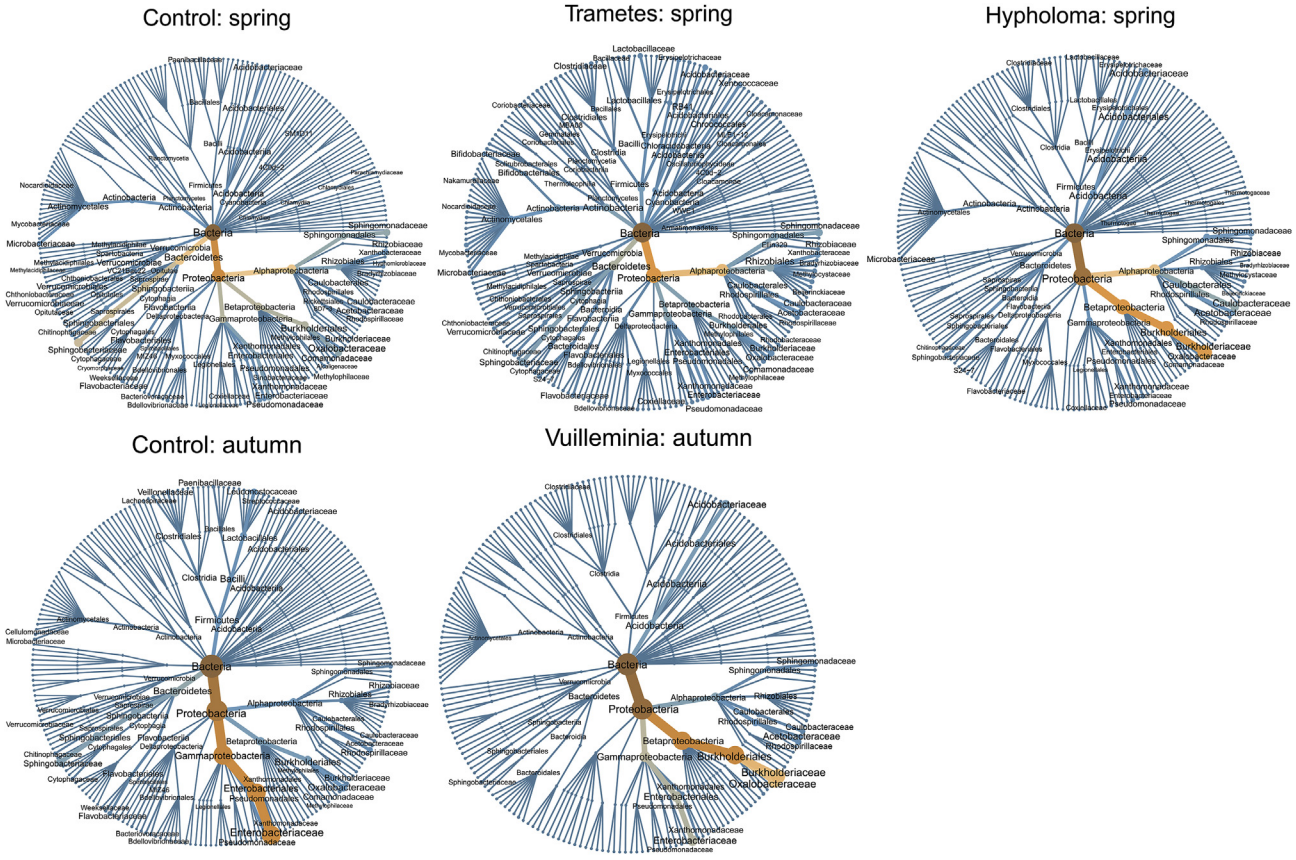

Fig. 5. Bacterial community composition in fungus-colonised and control wood blocks, broken down by pre-coloniser treatment and season. *T. versicolor* and *H. fasciculare* could only be analysed in the spring, *V. comedens* only in the autumn. All samples were incubated in the field for 84 d. Node colour and size on the heat trees represent relative abundance for that taxon; relative read abundance is on an arbitrary scale where each sample sums to 100. OTUs assigned to *Archaea* or unassigned at the Domain/Kingdom level are excluded for ease of visualisation.

community OTU richness to a similar extent. Given that *T. versicolor* was the fungus that most successfully prevented bacteria colonisation, it is surprising that the bacterial community associated with it was not different in richness or structure compared to the controls. One possible explanation is that it reflects the mechanism of bacterial exclusion. *T. versicolor* forms dense, rubbery mycelium around the outside of wood blocks. Although *T. versicolor* also produces a wide range of enzymes (Hiscox et al. 2010), if it mainly excluded bacteria via a physical, rather than chemical, barrier, this may explain why there was less discrimination between taxa.

The difference between control and pre-colonised wood samples demonstrated the important distinction between fresh and canopy-decayed wood, with several important implications. Firstly, experiments using sterile, undecayed wood to study bacterial colonisation should recognise that this represents a very small subset of wood naturally reaching the forest floor. Secondly,

senescent wood from unmanaged forests will follow a different path of bacterial colonisation than wood from felled trees and branches, typical of wood inputs in commercial forestry. Lower bacterial diversity and altered community composition have been recorded for *F. sylvatica* logs in comparisons of managed and unmanaged forests (Hoppe et al. 2015). Different successional paths have also been observed for fungal communities in fresh and pre-colonised wood, although very early decay ascomycetes complicate the situation (Hiscox et al., 2015, 2016).

Flattening of the rarefaction curves for pre-colonised samples indicated that the bacterial community was comprehensively sampled, and further evidences community simplification in the presence of fungi. This agrees with previous observations of reduced bacterial diversity in wood and soil environments under fungal influence (Folman et al. 2008; Boersma et al. 2009; Johnston et al. 2018). Hervé et al. (2014) recorded completely saturated bacterial rarefaction curves for wood even in the absence of fungi, but it is likely that their microcosms exerted selection effects on the bacterial community. Sun et al. (2014) reported highly unsaturated bacterial communities after 2 and 4 months of wood decay, but this discrepancy is likely because that study did not investigate pre-colonised wood and had far lower read numbers than the present study.

4.3. The early-colonising bacterial community differs from later stage communities

The timescale of bacterial wood colonisation is poorly explored (Sun et al., 2013, 2014; Hervé et al. 2014). After 14 d bacteria could be detected throughout a 3 cm cube of fresh sapwood, indicating that they were able to colonise wood rapidly under favourable conditions. In fresh pinewood, bacterial richness and diversity increased between 2 and 4 months, suggesting that not all the available niches had been occupied within that time frame (Sun et al. 2014). In contrast, a microcosm experiment found no effect of time or fungal colonisation on bacterial richness, and limited effects on community composition (Hervé et al. 2014). This likely reflects the somewhat artificial experimental set-up, where time was not measured on replicates of increasing age, but rather by repeated sub-culturing of the developing community.

Psuedomonadaceae declined through time in both seasons. A decrease in the relative abundance of pseudomonads has also been observed over longer times (Sun et al., 2013, 2014; Kielak et al. 2016). They were replaced by *Enterobacteriaceae* and *Sphingobacteriaceae* in autumn and spring, respectively. *Enterobacteriaceae* are known to inhabit decaying sapwood (Zhang et al. 2008), and some members possess lignocellulytic enzymes (Bugg et al. 2011). *Sphingobacteria* have also been previously reported in wood (Sun et al., 2013, 2014) and are known to associate with fungi (Warmink and van Elsas, 2009; Pent et al. 2017).

Comparing the present samples to wood pre-colonised by the same fungi but allowed to decay in the field for a year (Johnston et al. 2018) revealed *Burkholderiales* were important members of both early and established communities. In contrast, there was a large difference in the relative abundance of *Acidobacteria*. This phylum was one of the largest components of the bacterial community in the 1-y samples, yet a minor constituent in the early-decay community, suggesting a slow rate of colonisation. In concordance with this, *Acidobacteria* increased in relative abundance in decomposing wood over a timescale of 2–4 months (Sun et al. 2014), and between 1 y and 6 y, before decreasing again by 13 y (Sun et al. 2013). However, Hoppe et al. (2015) recorded no difference in the relative abundance of *Acidobacteria* in logs across multiple decay classes, and Tláskal et al. (2017) recorded them as a major taxon at all decay stages.

4.4. Seasonal influences are dependent on stage of bacterial community succession

Regrettably, the scarcity of bacteria-positive samples meant that prediction 3 (greater fungal influence in the autumn) could not be tested. However, the 84 d control samples revealed seasonal differences independent of the pre-colonisers. Fungal contribution cannot be ruled out, as the effect could be mediated by different incoming fungal communities between seasons (Hiscox et al. 2015). It has been postulated that during the early stages of wood colonisation, bacterial communities are determined by both neutral (stochastic) and niche-based processes, with niche-based processes becoming dominant at later stages of decay (Kielak et al. 2016). Intra-seasonal differences likewise could not be fully tested, but the autumn 84-d samples showed reduced diversity on one of the three collection dates. This sampling had been preceded by several days of hard frost, which may have adversely affected sections of the bacterial community.

4.5. Archaea are a minor but detectable part of the dead-wood prokaryote community

Consistent with previous reports from decaying wood, *Archaea* accounted for <1% of the prokaryote community (Rinta-Kanto et al. 2016). *Thaumarchaeota* were not represented, despite being the largest archaeal component in previous surveys of decaying wood (Rinta-Kanto et al. 2016). Archaeal PCR on the samples from Johnston et al. (2018) also found *Thaumarchaeota* to be the phylum that occurred most frequently (Johnston et al., unpubl. data). Their current absence may be attributable to a known bias against *Thaumarchaeota* in the 515F-806R primer set (Parada et al. 2016). *Methanobacteria* inhabit living trees as part of the phenomenon known as bacterial wetwood (Zeikus and Ward, 1974; Zeikus and Henning, 1975; Yip et al. 2019), and have also been found in decomposing logs (Moll et al. 2018). *Parvarchaeota* are putative acidophiles (Hedlund et al. 2014), which may explain their presence in the acidic dead-wood environment (Johnston et al. 2018).

5. Conclusions

In conclusion, this study provides strong evidence that wood-decay fungi act as 'gatekeepers', exerting control over which bacteria can colonise a woody resource. This effect is manifest both in delayed bacterial community development in fungus-colonised wood, and in modified bacterial community composition dependent on the identity of the fungal pre-coloniser. Fungal colonisation significantly delays the process of bacterial establishment, indicating that community development will differ between canopy-decayed and fresh wood. Even early-decay fungi with low competitive ability are capable of modifying the bacterial community. Bacterial community composition in wood varies between seasons, but only once the community is relatively established.

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Conflicts of interest

The authors declare no conflict of interest.

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Supplementary data

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